[Chem. Pharm. Bull. 33(8)3390—3394(1985)]

Mitochondrial Uptake of $^{45}Ca^{2+}$ Bound to Calcium-Binding Protein Isolated from Rat Liver Cytosol

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(Received November 15, 1984)

The mitochondrial uptake of $^{45}Ca^{2+}$ bound to calcium-binding protein newly isolated from rat liver cytosol was investigated. The binding of $^{45}Ca^{2+}$ to calcium-binding protein increased linearly with increasing amount of the protein. $^{45}Ca^{2+}$ bound to the binding protein was taken up by rat liver mitochondria and microsomes in the presence of 3 mM adenosine 5′-triphosphate (ATP) in the incubation medium, while the uptake was slight in the absence of ATP. The mitochondrial uptake of $^{45}Ca^{2+}$ bound to the binding protein started within 15 s of the start of incubation and was saturated at 5 min. The amount of $^{45}Ca^{2+}$ taken up by the mitochondria from $^{45}Ca^{2+}$-binding protein increased linearly with increasing concentration of the protein-bound $^{45}Ca^{2+}$. The mitochondrial uptake of $^{45}Ca^{2+}$ from the binding protein was markedly inhibited by the presence of mitochondrial calcium uptake inhibitors, ruthenium red (10 μM), lanthanum chloride (250 μM), and oxidized form of nicotinamide adenine dinucleotide (NAD^+; 2.5 mM). The present results suggest that the cytosolic calcium-binding protein binds calcium ion in rat liver cytosol and the metal is subsequently transported into the organelles. This protein may play a role in the regulation of the cytosolic calcium ion level.

Keywords——calcium-binding protein; calcium transport; mitochondrial uptake; microsomal uptake; rat liver cytosol

Introduction

Many biochemical effects of calcium ions in cells are mediated by a family of homologous calcium-binding protein.[1,2] Several such proteins, including calmodulin, calcineurin B, parvalbumin, S-100a, S-100b proteins and CBP-18, are mainly found in the brain.[3-7] Other calcium-binding proteins have been identified in the intestine, kidney and parathyroid glands in mammals, and in the eggshell gland of laying hens, where a large flux of calcium occurs.[8-11] Although the physiological role of these calcium-binding proteins remains to be elucidated, their high concentration in the above tissues suggests that they may be involved in transcellular transport of calcium ions.[12]

It has recently been demonstrated that the liver participates in the regulation of calcium metabolism by the hepatic bile system in rats.[13,14] In accordance with this finding, a calcium-binding protein has been found in the cytosol of rat liver; its purification and properties have been reported.[15,16] The molecular weight of the calcium-binding protein was estimated to be 28800, and the calcium binding constant was found to be $4.19 \times 10^5\ M^{-1}$ by equilibrium dialysis.[16] The physiological significance of the calcium-binding protein, however, has not been elucidated. The present report deals with the mitochondrial uptake of $^{45}Ca^{2+}$ bound to the calcium-binding protein isolated from rat liver cytosol.

Materials and Methods

Chemicals——$^{45}CaCl_2$ (specific activity 1 mCi/mmol) was obtained from New England Nuclear (Boston, Mass., U.S.A.), and ruthenium red, lanthanum chloride and oxidized form of nicotinamide adenine dinucleotide (NAD^+)

were from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). All other reagents were purchased from Wako Pure Chemical Co. (Osaka, Japan). Chelex-100 resin was obtained from Bio Rad (Richmond, Calf., U.S.A.).

Isolation of Calcium-Binding Protein——Calcium-binding protein in the cytosol fraction (supernatant of 105000 × *g* centrifugation) of rat liver was purified to electrophoretic homogeneity by gel filtration on Sephadex G-75 and G-50 followed by ion-exchange chromatography on diethylaminoethyl-cellulose, as reported previously.[15]

Preparation of $^{45}Ca^{2+}$-Binding Protein——Assay for $^{45}Ca^{2+}$ binding was based on the competitive-binding Chelex-100 method of Wasserman and Taylor.[8] Calcium-binding protein (10—100 μg) was added to test tubes in a volume of 1.0 ml, followed by 0.2 ml of Chelex in suspension. Then 0.1 ml of 10 μM Ca^{2+} solution containing $^{45}CaCl_2$ (0.5 μCi/ml) was added, and the tubes were incubated for 10 min at 4 °C, and centrifuged at 10500 × *g* for 10 min at 4 °C. The $^{45}Ca^{2+}$ radioactivity in 0.2 ml aliquots of the supernatant was determined by liquid scintillation spectrophotometry.[17] The blank preparation contained no calcium-binding protein.

Preparation of Rat Liver Mitochondria and Microsomes——The liver of male Wistar rats (weighing about 120 g) was perfused with an ice-cold 0.25 M sucrose solution, cut into small pieces, suspended 1 : 4 in 0.25 M sucrose solution and homogenized in a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was spun at 600 × *g* in a refrigerated centrifuge for 10 min and the supernatant was spun at 5500 × *g* for 20 min to obtain the mitochondria. The 5500 × *g* supernatant was spun at 105000 × *g* for 60 min to obtain the microsomes.[18] The mitochondria and microsomes were suspended in Tris–HCl buffer (pH 7.4, containing 10 mM Tris, 120 mM NaCl and 4 mM KCl, cooled to 4 °C) at a concentration of 3.0 mg of protein/ml and stored in an ice bath before use.

Measurement of $^{45}Ca^{2+}$ Uptake from $^{45}Ca^{2+}$-Binding Protein——Mitochondrial and microsomal $^{45}Ca^{2+}$ uptakes from $^{45}Ca^{2+}$-binding protein were measured by preincubating the organelles for 2 min at 4 and 37 °C in 1.0 ml of medium containing 10 mM Tris–HCl (pH 7.4), 120 mM NaCl and 3 mM ATP at a concentration of 3 mg of organelle protein/ml. The uptake was started by addition of $^{45}Ca^{2+}$-binding protein (50 μg/0.2 ml, 4629 ± 860 cpm) with gentle shaking unless otherwise specified. At defined intervals, the mixture was filtered through a Millipore disk (25 mm diameter; 0.45 μm pore size), which was then quickly washed twice with 10 ml of the incubation medium under suction. Radioactivity trapped on the filter disk was counted in a liquid scintillation spectrometer. The Millipore filter disk used in the present study had been coated with 1% polylysine or 0.05% methylglycolchitosan to minimize the radioactivity of filter blanks (nonspecific binding of $^{45}Ca^{2+}$ to the filter in the absence of mitochondria and microsomes), and data were corrected for the filter blank values. $^{45}Ca^{2+}$ uptake was expressed as a percent of the total radioactivity of $^{45}Ca^{2+}$-binding protein added to the medium.

In further experiments, the mitochondrial $^{45}Ca^{2+}$ uptake from $^{45}Ca^{2+}$-binding protein was measured in the presence of various inhibitors. At 10 min after the start of incubation of the mixture, 0.5 ml samples of the suspension were withdrawn and rapidly transferred to tubes containing 0.2 ml of medium supplemented with 2 mM ethylene glycol bis-(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), then centrifuged in a microcentrifuge at 12000 × *g* for 1.5 min. A portion of the supernatant was counted for radioactivity. The tubes were gently washed with 1.0 ml of ice-cold medium, and the pellet was resuspended with 0.5 ml of water. After addition of 0.5 ml of 20% (w/v) trichloroacetic acid, the tubes were centrifuged at 12000 × *g* for 2 min, and the supernatant was counted for radioactivity, which was considered to be due to intramitochondrial $^{45}Ca^{2+}$.

Protein concentration was determined by the method of Lowry *et al.*[19]

Statistical Methods——The results were subjected to analysis of variance, and the S.E. was calculated from the residual error term. Statistical significance is expressed in terms of *p* values from Student's *t*-test.

Results

The binding of $^{45}Ca^{2+}$ to calcium-binding protein isolated from rat liver cytosol was a linear function of the binding protein concentration up to 100 μg/ml (Fig. 1). Thus, increase in the radioactivity implies that $^{45}Ca^{2+}$ was bound to the protein, because free $^{45}Ca^{2+}$ in the incubation mixture was completely removed by addition of Chelex-100 resin. The amount of calcium bound to the protein was 3.78 nmol Ca per nmol protein. $^{45}Ca^{2+}$ bound to calcium-binding protein was transported into the mitochondria and microsomes prepared from rat liver homogenate, as evaluated by the Millipore filter method (Fig. 2). $^{45}Ca^{2+}$ uptake was unaltered by incubation temperature (4 or 37 °C). In an incubation medium without adenine 5′-triphosphate (ATP), $^{45}Ca^{2+}$ radioactivity taken up by the mitochondria and microsomes was about 20—30% of the total radioactivity of $^{45}Ca^{2+}$ on $^{45}Ca^{2+}$-binding protein added. The radioactivity was increased over 2-fold in the presence of 3 mM ATP in the incubation medium. The transport of free $^{45}Ca^{2+}$ into the mitochondria and microsomes was not facilitated by the presence of the binding protein in the incubation medium (data not shown).

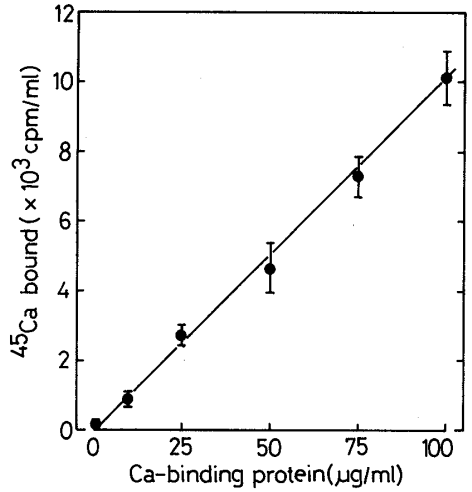

Fig. 1. Binding of $^{45}Ca^{2+}$ to Calcium-Binding Protein Isolated from Rat Liver Cytosol

Experimental details are given in Materials and Methods. Values are means ± S.E. for five experiments.

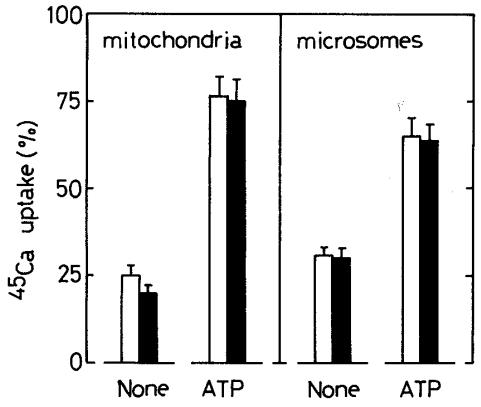

Fig. 2. The Mitochondrial and Microsomal Uptake of $^{45}Ca^{2+}$ Bound to Calcium-Binding Protein Isolated from Rat Liver Cytosol

Experimental details are given in Materials and Methods. Calcium-binding protein was used at a concentration of 50 μg/ml. The mitochondrial and microsomal $^{45}Ca^{2+}$ uptakes were observed in the absence and in the presence of 3 mM ATP for 10 min at 4 °C (□) and 37 °C (■). Values are means ± S.E. for five experiments.

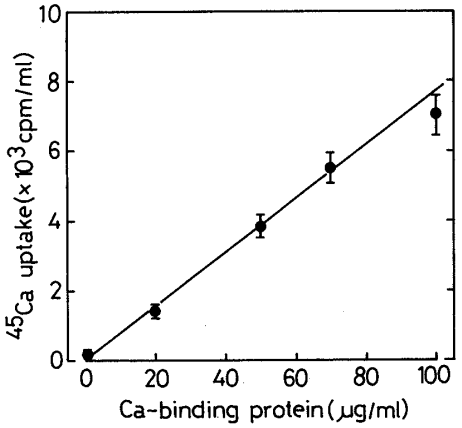

Fig. 3. Uptake of $^{45}Ca^{2+}$ by Mitochondria in Relation to the Amount of the Calcium-Binding Protein

Experimental details are given in Materials and Methods. The mitochondrial $^{45}Ca^{2+}$ uptake was observed in the presence of 3 mM ATP for 10 min at 37 °C. Values are means ± S.E. for five experiments.

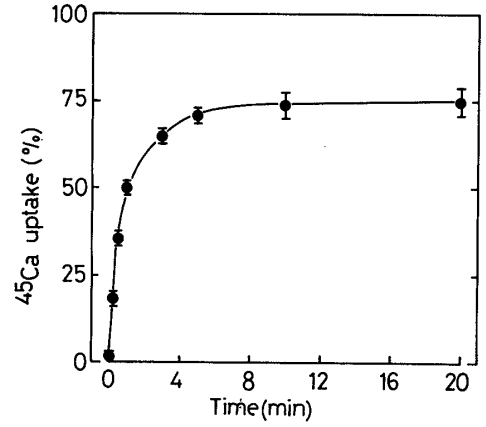

Fig. 4. Time Course of the Mitochondrial Uptake of $^{45}Ca^{2+}$ Bound to the Calcium-Binding Protein

Experimental details are given in Materials and Methods. Calcium-binding protein was used at a concentration of 50 μg/ml. The mitochondrial $^{45}Ca^{2+}$ uptake was observed in the presence of 3 mM ATP at 37 °C. Values are means ± S.E. for five experiments.

The transport of $^{45}Ca^{2+}$ bound to calcium-binding protein was investigated in detail in rat liver mitochondria, because it is now generally accepted that mitochondria play an important role as a store of cytosolic calcium. When $^{45}Ca^{2+}$ transport into the mitochondria was examined by the microcentrifuge method, the result was the same as that obtained by the Millipore filter method, indicating that the increase in radioactivity resulted from mitochondrial uptake, but not deposition of $^{45}Ca^{2+}$-binding protein on the filter. Further studies were performed by the microcentrifuge method. Figure 3 shows that the mitochondrial uptake of $^{45}Ca^{2+}$ from the binding protein was a linear function of the amount of protein binding $^{45}Ca^{2+}$, when mitochondria (3 mg protein/ml) were incubated with $^{45}Ca^{2+}$-binding protein in

TABLE I. Effects of Various Inhibitors on the Mitochondrial Uptake of $^{45}Ca^{2+}$ Bound to the Calcium-Binding Protein

Inhibitor added[a]	$^{45}Ca^{2+}$ uptake (%)[b]	
	4 °C	37 °C
None	70.1 ± 3.1	76.9 ± 2.9
1 μM ruthenium red	42.3 ± 3.1[c]	46.3 ± 2.9[c]
10 μM ruthenium red	37.1 ± 2.8[c]	41.9 ± 2.7[c]
25 μM lanthanum chloride	55.4 ± 1.3[c]	59.1 ± 2.5[c]
250 μM lanthanum chloride	9.2 ± 1.5[c]	4.7 ± 1.0[c]
0.5 mM NAD^+	69.5 ± 2.5[c]	71.4 ± 2.0[c]
2.5 mM NAD^+	5.0 ± 0.5[c]	12.8 ± 1.5[c]

a) Experimental details are given in Materials and Methods. $^{45}Ca^{2+}$-binding protein was used at a concentration of 50 μg/ml. The mitochondrial $^{45}Ca^{2+}$ uptake was observed in the presence of 3 mM ATP and various drugs for 10 min at 4 and 37 °C. *b*) Values are means ± S.E. for five experiments. *c*) $p < 0.01$, as compared with "none."

the presence of 3 mM ATP at 37 °C for 10 min. The percent of $^{45}Ca^{2+}$ taken up by the mitochondria was constant, as the amount of the binding protein was increased.

The time course of transport into the mitochondria of $^{45}Ca^{2+}$ from the binding protein is shown in Fig. 4. About 20% of $^{45}Ca^{2+}$ bound to the binding protein was transported into mitochondria during incubation for 15 s. The transport was rapid and reached the steady state within 5 min. However, the $^{45}Ca^{2+}$ uptake was not facilitated by the addition of calcium-binding protein. Thus, only $^{45}Ca^{2+}$ bound to the binding protein was clearly transported into the mitochondria.

The effects of various inhibitors on the transport into mitochondria of $^{45}Ca^{2+}$ from the binding protein were studied in the presence of 3 mM ATP (Table I). When ruthenium red was added to the incubation medium at 1 or 10 μM, the mitochondrial transport of $^{45}Ca^{2+}$ from the binding protein was markedly inhibited. Lanthanum chloride (250 μM) and NAD^+ (2.5 mM) also inhibited the mitochondrial transport of $^{45}Ca^{2+}$ from the binding protein. These inhibitions were essentially the same at both 4 and 37 °C.

Discussion

In recent years, it has been demonstrated that the liver participates in the regulation of calcium metabolism; calcium in the serum is transferred into the bile through the liver in rats.[13,14] Following this finding, it was found that calcium-binding protein exists in the cytosol of rat liver.[15,16] Amino acid analysis of this protein showed glycine and glutamic acid to be the predominant amino acids. The calcium binding constant was found to be $4.19 \times 10^5\ M^{-1}$ by equilibrium dialysis. It is assumed that this protein plays a role in cytosolic calcium ion regulation, because of the transcellular transport of calcium in rat liver, as described above. In the present work, it was examined whether or not calcium bound to the binding protein in the cytosol is transported into the organelles of rat liver cells.

The mitochondria or microsomes were incubated with $^{45}Ca^{2+}$-labeled calcium-binding protein in the absence of ATP. The increase in $^{45}Ca^{2+}$ radioactivity of the mitochondria and microsomes was slight. However, the mitochondria and microsomes could clearly take up $^{45}Ca^{2+}$ from calcium-binding protein in the presence of 3 mM ATP. This $^{45}Ca^{2+}$ uptake increased progressively with increasing amount of $^{45}Ca^{2+}$-binding protein. The results clearly indicate that $^{45}Ca^{2+}$ bound to the binding protein was transferred into the mitochondria and microsomes by a process dependent on ATP.

The transfer of $^{45}Ca^{2+}$ bound to the binding protein into liver mitochondria was markedly inhibited by the presence of ruthenium red, lanthanum chloride and NAD^+, which are potent inhibitors of mitochondrial calcium uptake.[20] The inhibition was unaffected by alteration of temperature (4 and 37 °C) in the presence of ATP. These results further support the hypothesis that $^{45}Ca^{2+}$ was passed from the $^{45}Ca^{2+}$-binding protein into the organelles, that the transfer of $^{45}Ca^{2+}$-binding protein itself into the mitochondria did not occur, and that the transport required a supply of energy.

The transfer of $^{45}Ca^{2+}$ from calcium-binding protein into liver mitochondria started within 15 s after addition of $^{45}Ca^{2+}$-binding protein in the presence of ATP, and increased rapidly up to 5 min of incubation. This result indicates that calcium ions bound to calcium-binding protein of hepatic cytosol pass easily and quickly into mitochondria. The cytosolic calcium-binding protein may thus prevent a rapid increase of calcium ion concentration in the cytosol by transferring the metal to the mitochondria and the microsomes for storage as intracellular calcium. In view of the many biochemical effects of calcium ion in liver cells,[21] and the finding that excess calcium in liver cells is excreted into the bile through the bile duct of rat liver,[14] the calcium-binding protein newly found in rat liver cytosol may play an important physiological role in regulating calcium ion concentration in the cytosol.

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